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(54) MONOOXYGENASES A CYTOCHROME-P450 ET LEUR UTILISATION POUR L'OXYDATION DE COMPOSES ORGANIQUES

(54) NOVEL CYTOCHROME P450 MONOOXYGENASES AND THEIR USE FOR OXIDIZING ORGANIC COMPOUNDS

(57)

The invention relates to novel cytochrome P450 monooxygenases comprising a modified substrate specificity, to nucleotide sequences which code therefor, to expression constructs and vectors containing these sequences, and to microorganisms transformed therewith. The invention also relates to methods for microbiologically oxidizing different organic substrates, such as methods for producing indigo and indirubin.



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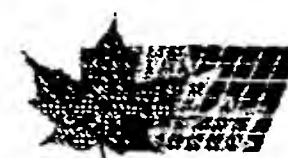
The invention relates to novel cytochrome P450 monooxygenases comprising a modified substrate specificity, to nucleotide sequences which code therefor, to expression constructs and vectors containing these sequences, and to microorganisms transformed therewith. The invention also relates to methods for microbiologically oxidizing different organic substrates, such as methods for producing indigo and indirubin.

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VON ORGANISCHEN VERBINDUNGEN(57) Abstract: The invention relates to novel cytochrome P450 monooxygenases comprising a modified substrate specificity, to
nucleotide sequences which code therefor, to expression constructs and vectors containing these sequences, and to microorganisms
transformed therewith. The invention also relates to methods for microbiologically oxidizing different organic substrates, such as
methods for producing indigo and indirubin.(57) Zusammenfassung: Die Erfindung betrifft neue Cytochrom P450-Monooxygenasen mit veränderter Substratspezifität, dafür
kodierende Nukleotidsequenzen, diese Sequenzen enthaltende Expressionskonstrukte und Vektoren, damit transformierte Mikroor-
ganismen, Verfahren zur mikrobiologischen Oxidation verschiedener organischer Substrate wie beispielsweise Verfahren zur Her-
stellung von Indigo und Indirubin.

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NOVEL CYTOCHROME P450 MONOOXYGENASES AND THEIR
USE FOR OXIDIZING ORGANIC COMPOUNDS

The present invention relates to novel cytochrome P450 monooxygenases with modified substrate specificity which are capable of the oxidation of organic substrates, for example N-heterocyclic aromatic compounds, nucleotide sequences coding therefor, expression constructs and vectors comprising these sequences, microorganisms transformed therewith, processes for the microbiological oxidation of various organic substrates, such as N-heterocyclic aromatic compounds and in particular processes for the preparation of indigo and indirubin.

- 10 Enzymes having novel functions and properties can be prepared either by screening of natural samples or by protein engineering of known enzymes. Under certain circumstances, the last-mentioned method can be the more suitable to induce characteristics whose generation by the natural selection route is improbable. Despite numerous attempts at the engineering of enzymes, up to now there are only a few successful studies for promoting the catalytic activity of enzyme mutants with respect to a certain substrate (1-10). In these known cases, the substrates are structurally closely related to the native substrate of the respective enzyme. As yet, there are no reports on the successful engineering of enzymes which, after modification, catalyze the reaction of a compound which structurally is completely different from the native substrate of the enzyme.

- 20 The cytochrome P450 monooxygenase isolatable from the bacterium *Bacillus megaterium* usually catalyzes the subterminal hydroxylation of long-chain, saturated acids and the corresponding amides and alcohols thereof or the epoxidation of unsaturated long-chain fatty acids or saturated fatty acids of medium chain length (11-13). The optimal chain length of saturated fatty acids is 14 to 16 carbon atoms. Fatty acids having a chain length of less than 12 are not hydroxylated (11).

- 30 The structure of the heme domain of P450 BM-3 was determined by X-ray structural analysis (14-16). The substrate binding site is present in the form of a long tunnel-like opening which extends from the surface of the molecule as far as the heme molecule and is almost exclusively bordered by hydrophobic amino acid residues. The only charged residues on the surface of the heme domain are the residues Arg47 and Tyr51. It is assumed that these are involved in the binding of the carboxylate group of the substrate by formation of a hydrogen bond (14). The mutation of

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Arg47 to Glu brings about inactivation of the enzyme for arachidonic acid (13), but increases its activity compared with C₁₂-C₁₄-alkyltrimethylammonium compounds (17). Substrate utilization for aromatic compounds, in particular mono-, bi- or polynuclear, if desired heterocyclic, aromatics, alkanes, alkenes, cycloalkanes and cycloalkenes, has not been described for this enzyme. Until now, it was therefore assumed in specialist circles that substrates other than the organic substrates hitherto described, for example indole, on account of the clear structural differences from the native substrates of P450 BM-3, in particular on account of the absence of functional groups which could bind to the abovementioned residues in the substrate pocket, are not a substrate.

It is an object of the present invention to make available novel cytochrome P450 monooxygenases having modified substrate specificity or modified substrate profile. In particular, monooxygenase mutants are to be provided which, in comparison with the nonmutated wild-type enzyme, are enzymatically active with structurally clearly different substrates.

Compared to the wild-type enzyme, a "modified substrate profile" can be observed for the mutants according to the invention. In particular, for the mutant in question, an improvement in reactivity is observed, for example an increase of the specific activity (expressed as nmol of converted substrate/minute/nmol of P450 enzyme) and/or of at least one kinetic parameter selected from the group consisting of K_{cat}, K_m and K_{cat}/K_m (for example by at least 1%, such as 10 to 1000%, 10 to 500% or 10 to 100%) in the conversion of at least one of the oxidizable compounds defined in groups a) to d). The oxidation reaction according to the invention comprises the enzyme-catalyzed oxygenation of at least one exogenous (i.e. added to the reaction medium) or endogenous (i.e. already present in the reaction medium) organic substrate. In particular, the oxidation reaction according to the invention comprises a mono- and/or polyhydroxylation, for example a mono- and/or dihydroxylation, at an aliphatic or aromatic C-H group, or an epoxidation at a C=C group which is preferably non-aromatic. Also possible are combinations of the above reactions. Moreover, the immediate reaction product can be converted further in the context of a non-enzymatic subsequent or side reaction. Such combinations of enzymatic and non-enzymatic processes likewise form part of the subject-matter of the present invention.

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We have found that the above object is surprisingly achieved by means of novel cytochrome P450 monooxygenases which, for example, are capable of the oxidation of N-heterocyclic bi- or polynuclear aromatic compounds.

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In particular, the invention relates to those monooxygenases whose substrate-binding region is capable by means of site-specific mutagenesis of the functional uptake of novel, for example N-heterocyclic substrates.

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In a preferred embodiment of the invention, the novel monooxygenases are soluble, i.e. existent in non membrane-bound form, and enzymatically active in this form.

- 15 The monooxygenases according to the invention are preferably derived from cytochrome P450 monooxygenases of bacterial origin, as derived, in particular, from cytochrome P450 monooxygenase BM-3 from *Bacillus megaterium* having an amino acid sequence according to SEQ ID NO:2, which has at least one functional
- 20 mutation, i.e. promoting the oxidation of novel organic substrates (cf. in particular the groups a) to d) of compounds as defined below), for example N-heterocyclic mono-, bi- or polynuclear aromatic compounds, in one of the amino acid sequence regions 172-224 (F/G loop region), 39-43 (β -strand 1), 48-52
- 25 (β -strand 2), 67-70 (β -strand 3), 330-335 (β -strand 5), 352-356 (β -strand 8), 73-82 (helix 5) and 86-88 (helix 6).

The cytochrome P450 monooxygenase mutants provided according to the invention are preferably capable of at least one of the

30 following reactions:

- a) oxidation of unsubstituted or substituted N-, O- or S-heterocyclic mono-, bi- or polynuclear aromatic compounds;
- b) oxidation of unsubstituted or substituted mono- or
- 35 polynuclear aromatics;
- c) oxidation of straight-chain or branched alkanes and alkenes; and
- d) oxidation of unsubstituted or substituted cycloalkanes and cycloalkenes.

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Preferred monooxygenase mutants have at least one functional mutation, in particular amino acid substitution, in at least one of the sequence regions 73-82, 86-88 and 172-224. Thus, for example, Phe87 can be replaced by an amino acid having an

45 aliphatic side chain, such as Ala, Val, Leu, in particular Val; Leu188 can be replaced by an amino acid having an amide side chain, such as Asn or, in particular, Gln; and Ala74 can be

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replaced by another amino acid having an aliphatic side chain, such as Val and, in particular, Gly.

Particularly preferred monooxygenase mutants of this type are those which have at least one of the following mono- or polyamino acid substitutions:

- 1) Phe87Val;
- 2) Phe87Val, Leu188Gln; or
- 10 3) Phe87Val, Leu188Gln, Ala74Gly;

and functional equivalents thereof. The number indicates the position of the mutation; the original amino acid is indicated before the number and the newly introduced amino acid after the number.

In this context, "functional equivalents" or analogs of the mutants which are disclosed specifically are mutants differing therefrom which furthermore have the desired substrate specificity with respect to at least one of the oxidation reactions a) to d) described above, i.e., for example, for heterocyclic aromatics and which hydroxylate, for example, indole, or furthermore exhibit the desired "modified substrate profile" with respect to the wild-type enzyme.

"Functional equivalents" are also to be understood as meaning in accordance with the invention mutants which exhibit, in at least one of the abovementioned sequence positions, an amino acid substitution other than the one mentioned specifically, but still lead to a mutant which, like the mutant which has been mentioned specifically, show a "modified substrate profile" with respect to the wild-type enzyme and catalyze at least one of the abovementioned oxidation reactions. Functional equivalence exists in particular also in the case where the modifications in the substrate profile correspond qualitatively, i.e. where, for example, the same substrates are converted, but at different rates.

"Functional equivalents" naturally also encompass P450 monooxygenase mutants which, like the P450 BM3 mutants which have been mentioned specifically, can be obtained by mutating P450 enzymes from other organisms. For example, regions of homologous sequence regions can be identified by sequence comparison. Following the principles of what has been set out specifically in the invention, the modern methods of molecular modeling then

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allow equivalent mutations to be carried out which affect the reaction pattern.

"Functional equivalents" also encompass the mutants which can be obtained by one or more additional amino acid additions, substitutions, deletions and/or inversions, it being possible for the abovementioned additional modifications to occur in any sequence position as long as they give rise to a mutant with a modified substrate profile in the above sense.

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Substrates of group a) which can be oxidized according to the invention are unsubstituted or substituted heterocyclic mono-, bi- or polynuclear aromatic compounds; in particular oxidizable or hydroxylatable N-, O- or S-heterocyclic mono-, bi- or polynuclear aromatic compounds. They include preferably two or three, in particular two, 4- to 7-membered, in particular 6- or 5-membered, fused rings, where at least one, preferably all, rings have aromatic character and where at least one of the aromatic rings carries one to three, preferably one, N-, O- or S-heteroatom in the ring. The total ring structure may contain one or two further identical or different heteroatoms. The aromatic compounds may furthermore carry 1 to 5 substituents at the ring carbon or heteroatoms. Examples of suitable substituents are C₁- to C₄-alkyl, such as methyl, ethyl, n- or isopropyl, n-, iso- or t-butyl, or C₂- to C₄-alkenyl, such as ethenyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl or 3-butenyl, hydroxyl and halogen, such as F, Cl and Br. The alkyl or alkenyl substituents mentioned may also have a keto or aldehyde group; examples being propan-2-on-3-yl, butan-2-on-4-yl, 3-buten-2-on-4-yl. Non-limiting examples of suitable heterocyclic substrates are, in particular, binuclear heterocycles, such as indole, N-methyl-indole, and the substituted analogs thereof which carry one to three of the above-defined substituents on carbon atoms, for example 5-chloro- or 5-bromoindole; and also quinoline and quinoline derivatives, for example 8-methylquinoline, 6-methyl-quinoline and quinaldine; and benzothiophene, and the substituted analogs thereof which carry one to three of the above-defined substituents on carbon atoms. Moreover, trinuclear hetero-aromatics, such as acridine and the substituted analogs thereof which carry one to three of the above-defined substituents on carbon atoms, may be mentioned.

Substrates of group b) which are oxidizable according to the invention are unsubstituted or substituted mono- or polynuclear, in particular mono- or binuclear, aromatics, such as benzene and naphthalene. The aromatic compounds may be unsubstituted or mono- or polysubstituted and, for example, carry 1 to 5 substituents on

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the ring carbon atoms. Examples of suitable substituents are C₁- to C₄-alkyl, such as methyl, ethyl, n- or isopropyl or n-, iso- or t-butyl, or C₂- to C₄-alkenyl, such as ethenyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl or 3-butenyl, hydroxyl and
5 halogen, such as F, Cl and Br. The alkyl or alkenyl substituents mentioned may also have a keto or aldehyde group; Examples being propan-2-on-3-yl, butan-2-on-4-yl, 3-buten-2-on-4-yl. The aromatic may be fused with a four- to seven-membered non-aromatic ring. The non-aromatic ring may have one or two C=C double bonds,
10 be mono- or polysubstituted by the abovementioned substituents and may carry one or two hetero ring atoms. Examples of particularly suitable aromatics are mononuclear aromatics, such as cumene, and binuclear substrates, such as indene and naphthalene, and substituted analogs thereof which carry one to
15 three of the above-defined substituents on carbon atoms.

Substrates of group c) which can be oxidized according to the invention are straight-chain or branched alkanes or alkenes having 4 to 15, preferably 6 to 12, carbon atoms. Examples which
20 may be mentioned are n-butane, n-pentane, n-hexane, n-heptane, n-octane, n-nonane, n-decane, n-undecane and n-dodecane, and the analogs of these compounds which are branched once or more than once, for example analogous compounds having 1 to 3 methyl side groups; or mono- or polyunsaturated, for example
25 mono-unsaturated, analogs of the abovementioned alkanes.

Substrates of group d) which can be oxidized according to the invention are unsubstituted or substituted cycloalkanes and cycloalkenes having 4 to 8 ring carbon atoms. Examples of these
30 are cyclopentane, cyclopentene, cyclohexane, cyclohexene, cycloheptane and cycloheptene. The ring structure may carry one or more, for example 1 to 5, substituents according to the above definition for compounds of groups a) and b). Nonlimiting examples are ionones, such as α -, β - and γ -ionone, and the
35 corresponding methyl ionones and iso-methyl ionones. Particular preference is given to α - and β -ionone.

The invention also relates to nucleic acid sequences coding for one of the monooxygenases according to the invention. Preferred
40 nucleic acid sequences are derived from SEQ ID NO:1, which have at least one nucleotide substitution which leads to one of the functional amino acid mutations described above. The invention moreover relates to functional analogs of the nucleic acids obtained by addition, substitution, insertion and/or deletion of
45 individual or multiple nucleotides, which furthermore code for a

monooxygenase having the desired substrate specificity, for example having indole-oxidizing activity.

The invention also encompasses those nucleic acid sequences which
5 comprise so-called silent mutations or which are modified in comparison with a specifically mentioned sequence in accordance with the codon usage of a specific origin or host organism, and naturally occurring variants of such nucleic acid sequences. The invention also encompasses modifications of the nucleic acid
10 sequences obtained by degeneration of the genetic code (i.e. without any changes in the corresponding amino acid sequence) or conservative nucleotide substitution (i.e. the corresponding amino acid is replaced by another amino acid of the same charge, size, polarity and/or solubility), and sequences modified by
15 nucleotide addition, insertion, inversion or deletion, which sequences encode a monooxygenase according to the invention having a "modified substrate profile", and the corresponding complementary sequences.

20 The invention furthermore relates to expression constructs comprising a nucleic acid sequence encoding a mutant according to the invention under the genetic control of regulatory nucleic acid sequences; and vectors comprising at least one of these expression constructs.

25 Preferably, the constructs according to the invention encompass a promoter 5'-upstream of the encoding sequence in question and a terminator sequence 3'-downstream, and, optionally, further customary regulatory elements, and, in each case operatively
30 linked with the encoding sequence. Operative linkage is to be understood as meaning the sequential arrangement of promoter, encoding sequence, terminator and, if appropriate, other regulatory elements in such a manner that each of the regulatory elements can fulfill its intended function on expression of the
35 encoding sequence. Examples of operatively linkable sequences are targeting sequences, or else translation enhancers, enhancers, polyadenylation signals and the like. Further regulatory elements encompass selectable markers, amplification signals, replication origins and the like.

40 In addition to the artificial regulatory sequences, the natural regulatory sequence can still be present upstream of the actual structural gene. If desired, this natural regulation may be switched off by genetic modification, and the expression of the
45 genes may be enhanced or lowered. However, the gene construct may also be simpler in construction, i.e. no additional regulatory signals are inserted upstream of the structural gene and the

natural promoter with its regulation is not removed. Instead, the natural regulatory sequence is mutated in such a way that regulation no longer takes place and the gene expression is increased or reduced. One or more copies of the nucleic acid sequences may be present in the gene construct.

Examples of suitable promoters are: cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, lacIq, T7, T5, T3, gal, trc, ara, SP6, 1-PR or 1-PL promoter, which are advantageously employed in Gram-negative bacteria; and Gram-positive promoters amy and SPO2, the yeast promoters ADC1, MFA, Ac, P-60, CYC1, GAPDH or the plant promoters CaMV/35S, SSU, OCS, lib4, usp, STLS1, B33, nos or the ubiquitin or phaseolin promoter. Particular preference is given to using inducible promoters, for example light- and in particular temperature-inducible promoters, such as the P_{rP_1} promoter.

In principle, all natural promoters with their regulatory sequences can be used. In addition, synthetic promoters may also be used in an advantageous fashion.

The abovementioned regulatory sequences are intended to allow the targeted expression of the nucleic acid sequences and of protein expression. Depending on the host organism, this may mean, for example, that the gene is expressed or overexpressed only after induction has taken place, or that it is expressed and/or overexpressed immediately.

The regulatory sequences or factors can preferably have a positive effect on expression and in this manner increase or reduce the latter. Thus, an enhancement of the regulatory elements may advantageously take place at the transcriptional level by using strong transcription signals such as promoters and/or "enhancers". In addition, translation may also be enhanced by improving, for example, mRNA stability.

An expression cassette is generated by fusing a suitable promoter with a suitable monooxygenase nucleotide sequence and a terminator signal or polyadenylation signal. To this end, customary recombination and cloning techniques are used as they are described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in

Molecular Biology, Greene Publishing Assoc. and Wiley Interscience (1987).

For expression in a suitable host organism, the recombinant
5 nucleic acid construct or gene construct is advantageously
inserted into a host-specific vector which allows optimal gene
expression in the host. Vectors are well known to the skilled
worker and can be found, for example, in "Cloning Vectors"
(Pouwels P.H. et al., Ed., Elsevier, Amsterdam-New York-Oxford,
10 1985). Vectors are to be understood as meaning not only plasmids,
but all other vectors known to the skilled worker such as, for
example, phages, viruses, such as SV40, CMV, baculovirus and
adenovirus, transposons, IS elements, phasmids, cosmids, and
linear or circular DNA. These vectors can be replicated
15 autonomously in the host organism or chromosomally.

The vectors according to the invention allow the generation of
recombinant microorganisms which are transformed, for example,
with at least one vector according to the invention and which can
20 be employed for producing the mutants. The above-described
recombinant constructs according to the invention are
advantageously introduced into a suitable host system and
expressed. It is preferred to use usual cloning and transfection
methods known to the skilled worker in order to bring about
25 expression of the abovementioned nucleic acids in the expression
system in question. Suitable systems are described, for example,
in current protocols in molecular biology, F. Ausubel et al.,
Ed., Wiley Interscience, New York 1997.

30 Suitable host organisms are, in principle, all organisms which
allow expression of the nucleic acids according to the invention,
their allelic variants, and their functional equivalents or
derivatives. Host organisms are to be understood as meaning, for
example, bacteria, fungi, yeasts or plant or animal cells.
35 Preferred organisms are bacteria such as those of the genera
Escherichia, such as, for example, Escherichia coli,
Streptomyces, Bacillus or Pseudomonas, eukaryotic microorganisms
such as Saccharomyces cerevisiae, Aspergillus, and higher
eukaryotic cells from animals or plants, for example Sf9 or CHO
40 cells.

If desired, expression of the gene product may also be brought
about in transgenic organisms such as transgenic animals such as,
in particular, mice, sheep, or transgenic plants. The transgenic
45 organisms may also be knock-out animals or plants in which the

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corresponding endogenous gene has been eliminated, such as, for example, by mutation or partial or complete deletion.

5 Successfully transformed organisms can be selected by marker genes which are likewise contained in the vector or in the expression cassette. Examples of such marker genes are genes for resistance to antibiotics and for enzymes which catalyze a color reaction, which causes staining of the transformed cell. These transformed cells can then be selected using automatic cell
10 selection. Microorganisms which have been transformed successfully with a vector and which carry an appropriate gene for resistance to antibiotics (for example G418 or hygromycin) can be selected by using appropriate antibiotics-containing media or substrates. Marker proteins which are presented on the cell
15 surface can be used for selection by affinity chromatography.

The combination of the host organisms and the vectors appropriate for the organisms, such as plasmids, viruses or phages, such as, for example, plasmids with the RNA polymerase/promoter system,
20 phages λ , μ or other temperate phages or transposons and/or other advantageous regulatory sequences forms an expression system. The term "expression system" means, for example, a combination of mammalian cells such as CHO cells, and vectors, such as pcDNA3neo vector, which are suitable for mammalian cells.

25 As described above, the gene product can also be expressed advantageously in transgenic animals, for example mice, sheep, or transgenic plants. It is likewise possible to program cell-free translation systems with the RNA derived from the nucleic acid.

30 The invention furthermore provides a process for preparing a monooxygenase according to the invention, which comprises cultivating a monooxygenase-producing microorganism, if appropriate inducing the expression of the monooxygenase, and
35 isolating the monooxygenase from the culture. If desired, the monooxygenase according to the invention can thus also be produced on an industrial scale.

The microorganism can be cultivated and fermented by known
40 methods. Bacteria, for example, can be grown in a TB or LB medium and at 20-40°C and a pH of 6-9. Suitable cultivation conditions are described in detail in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), for example.

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- If the monooxygenase is not secreted into the culture medium, the cells are then lyzed and the monooxygenase is obtained from the lysate using known methods for the isolation of proteins. The cells can be lyzed alternatively by high-frequency ultrasound, by
5 high pressure, for example in a French pressure cell, by osmolysis, by the action of detergents, lytic enzymes or organic solvents, by homogenization or by a combination of a plurality of the processes mentioned. Purification of the monooxygenase can be achieved by known chromatographic processes, such as molecular
10 sieve chromatography (gel filtration), such as Q-Sepharose chromatography, ion-exchange chromatography and hydrophobic chromatography, and by other customary processes, such as ultrafiltration, crystallization, salting out, dialysis and native gel electrophoresis. Suitable processes are described, for
15 example, in Cooper, F.G., Biochemische Arbeitsmethoden [Biochemical Procedures], Verlag Walter de Gruyter, Berlin, New York or in Scopes, R., Protein Purification, Springer Verlag, New York, Heidelberg, Berlin.
- 20 To isolate the recombinant protein, it is particularly advantageous to use vector systems or oligonucleotides which extend the cDNA by certain nucleotide sequences and thus code for modified polypeptides or fusion proteins which serve to simplify purification. Suitable modifications of this type are, for
25 example, so-called "tags" which act as anchors, such as, for example, the modification known as hexa-histidine anchor, or epitopes which can be recognized as antigens by antibodies (described, for example, in Harlow, E. and Lane, D., 1988, Antibodies: A Laboratory Manual. Cold Spring Harbor (N.Y.)
30 Press). These anchors can be used to attach the proteins to a solid support such as, for example, a polymer matrix, which can, for example, be packed into a chromatography column, or to a microtiter plate or to another support.
- 35 These anchors can also at the same time be used to recognize the proteins. It is also possible to use for recognition of the proteins conventional markers such as fluorescent dyes, enzyme markers which form a detectable reaction product after reaction with a substrate, or radioactive markers, alone or in combination
40 with the anchors for derivatizing the proteins.

The invention moreover relates to a process for the microbiological oxidation of organic compounds, for example N-heterocyclic mono-, bi- or polynuclear aromatic compounds
45 according to the above definition, which comprises

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- 5 a1) culturing a recombinant microorganism according to the above definition in a culture medium, in the presence of an exogenous (added) substrate or an intermediately formed substrate, which substrate is oxidizable by the monooxygenase according to the invention, preferably in the presence of oxygen (i.e. aerobically); or
- a2) incubating a substrate-containing reaction medium with an enzyme according to the invention, preferably in the presence of oxygen and an electron donor; and
- 10 b) isolating the oxidation product formed or a secondary product thereof from the medium.

The oxygen required for the reaction either passes from the atmosphere into the reaction medium or, if required, can be added
15 in a manner known per se.

The oxidizable substrate is preferably selected from

- 20 a) unsubstituted or substituted N-heterocyclic mono-, bi- or polynuclear aromatic compounds;
- b) unsubstituted or substituted mono- or polynuclear aromatics;
- c) straight-chain or branched alkanes and alkenes;
- d) unsubstituted or substituted cycloalkanes and cycloalkenes.

25 A preferred process variant is directed to the formation of indigo/indirubin and is characterized by the fact that the substrate is indole formed as an intermediate in the culture and that the indigo and/or indirubin formed in the culture medium is isolated by oxidation of hydroxyindole intermediates.

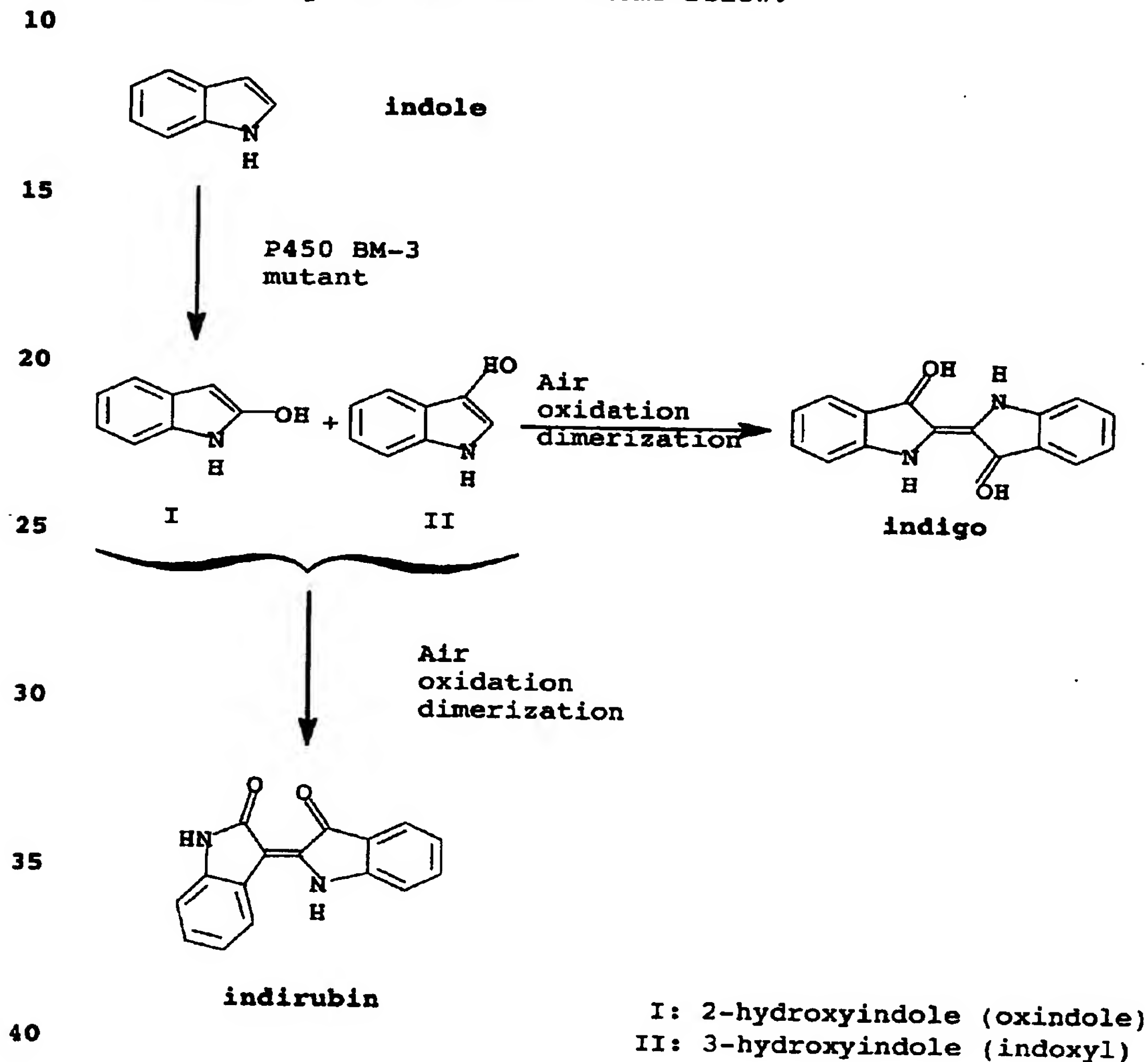
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If the oxidation according to the invention is carried out using a recombinant microorganism, the culturing of the microorganisms is preferably first carried out in the presence of oxygen and in a complex medium, such as, for example, TB or LB medium at a
35 culturing temperature of approximately 20 to 40°C and a pH of approximately 6 to 9, until an adequate cell density is reached. The addition of exogenous indole is usually not necessary, as this is intermediately formed by the microorganism. However, when using other substrates, addition of exogenous substrate may be
40 required. In order to be able to control the oxidation reaction better, the use of an inducible, in particular temperature-inducible, promoter is preferred. The temperature is in this case increased to the necessary induction temperature, e.g. 42°C in the case of the P_{rP1} promoter, this is maintained for a sufficient
45 period of time, e.g. 1 to 10 or 5 to 6 hours, for the expression of the monooxygenase activity and the temperature is then reduced again to a value of approximately 30 to 40°C. The culturing is

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then continued in the presence of oxygen for 12 hours to 3 days. The pH can, in particular in the case of indole oxidation, be increased by addition of NaOH, e.g. to 9 to 10, whereby the indigo formation or indirubin formation is additionally promoted by atmospheric oxidation of the enzymatically formed oxidation products 2- and 3-hydroxyindole.

The indigo/indirubin formation according to the invention is illustrated by the reaction scheme below:



45 However, if the oxidation according to the invention is carried out using purified or enriched enzyme mutants, the enzyme according to the invention is dissolved in an exogenous

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substrate-containing, for example indole-containing medium (approximately 0.01 to 10 mM, or 0.05 to 5 mM), and the reaction is carried out, preferably in the presence of oxygen, at a temperature of approximately 10 to 50°C, such as, for example, 30 5 to 40°C, and a pH of approximately 6 to 9 (such as established, for example, using 100 to 200 mM phosphate or tris buffer), and in the presence of a reductant, the substrate-containing medium moreover containing, relative to the substrate to be oxidized, an approximately 1- to 100-fold or 10- to 100-fold molar excess of 10 reduction equivalents. The preferred reductant is NADPH. If required, the reducing agent can be added in portions.

In a similar manner, the oxidizable substrates which are preferably used are: n-hexane, n-octane, n-decane, n-dodecane, 15 cumene, 1-methylindole, 5-Cl- or Br-indole, indene, benzothiophene, α -, β - and γ -ionone, acridine, naphthalene, 6-methyl- or 8-methylquinoline, quinoline and quinaldine.

The enzymatic oxidation reaction according to the invention can 20 be carried out, for example, under the following conditions:

Substrate concentration:	from 0.01 to 20 mM
Enzyme concentration:	from 0.1 to 10 mg/ml
25 Reaction temperature:	from 10 to 50°C
pH:	from 6 to 8
30 Buffer:	from 0.05 to 0.2 M potassium phosphate, or Tris/HCl
Electron donor:	is preferably added in portions (initial concentration about 0.1 to 2 mg/ml)
35	

The mixture can briefly (from 1 to 5 minutes) be preincubated (at about 20-40°C) before the reaction is initiated, for example by adding the electron donors (e.g. NADPH). The reaction is carried 40 out aerobically, if appropriate with additional introduction of oxygen.

In the substrate oxidation process according to the invention, oxygen which is present in or added to the reaction medium is 45 cleaved reductively by the enzyme. The required reduction

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equivalents are provided by the added reducing agent (electron donor).

The oxidation product formed can then be separated off from the medium and purified in a conventional manner, such as, for example, by extraction or chromatography.

Further subjects of the invention relate to bioreactors, comprising an enzyme according to the invention or a recombinant microorganism according to the invention in immobilized form.

A last subject of the invention relates to the use of a cytochrome P450 monooxygenase according to the invention or of a vector or microorganism according to the invention for the microbiological oxidation of a substrate from one of the groups a) to d), in particular of N-heterocyclic mono-, bi- or polynuclear aromatic compounds, and preferably for the formation of indigo and/or indirubin.

The present invention is now described in greater detail with reference to the following examples.

Example 1:

Randomization of specific codons of P450 BM-3

The experiments were carried out essentially as described in (19). Three positions (Phe87, Leu188 and Ala74) were randomized with the aid of site-specific mutagenesis using the Stratagene QuikChange kit (La Jolla, CA, USA). The following PCR primers were used for the individual positions:

Phe87: 5'-gcaggagacgggttgnnnacaagctggacg-3' (SEQ ID NO:3),
5'-cgtccagcttgtnnncaaccgctctcctgc-3', (SEQ ID NO:4)
Leu188: 5'-gaagcaatgaacaagnnnacgcgagcaaatccag-3' (SEQ ID NO:5),
5'-ctggatttgctcgcctgtnnncttgttcattgcttc-3' (SEQ ID NO:6);
Ala74: 5'-gctttgataaaaacttaagtcannnncttaaattgtacg-3' (SEQ ID:
NO:7),
5'-cgtacaaatttaagnnnnttgacttaagtttttatcaaagc-3' (SEQ ID
NO:8)

The conditions for the PCR were identical for all three positions. In particular, 17.5 pmol of one of each primer, 20 pmol of template plasmid DNA, 3 U of the Pfu polymerase and 3.25 nmol of each dNTP were used per 50 µl reaction volume. The PCR reaction was started at 94°C/1 min and the following temperature cycle was then carried out 20 times: 94°C, 1 min;

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46°C, 2.5 min; 72°C, 17 min. After 20 cycles, the reaction was continued at 72°C for 15 min. After the PCR, the template DNA was digested at 37°C for 3 h using 20 U of DpnI. *E. coli* DH5 α was then transformed. The transformed *E. coli* DH5 α cells were plated out 5 onto LB agar plates which contained 150 μ g/ml of ampicillin. Incubation was then carried out at 37°C for 18 h.

Example 2:

Expression and purification of the P450 BM-3 and its mutants and 10 production of a blue pigment

The P450 BM-3 gene and the mutants thereof were expressed under the control of the strong, temperature-inducible P_{RPL} promoter of the plasmid pCYTEXP1 in *E. coli* DH5 α as already described (20). 15 Colonies were picked up using sterile toothpicks and transferred to microtiter plates having 96 hollows, comprising 200 μ l of TB medium and 100 μ g/ml of ampicillin per hollow. Incubation was then carried out at 37°C overnight. 40 μ l of the cell culture of one of each hollow were then transferred to a culture tube which 20 contained 2 ml of TB medium with 100 μ g/ml of ampicillin. Culturing was then carried out at 37°C for 2 h. The temperature was then increased to 42°C for 6 h for induction. Culturing was then continued at 37°C overnight, a blue pigment being produced.

25 The preparative production of enzyme or blue pigment was carried out starting from a 300 ml cell culture ($OD_{578nm} = 0.8$ to 1.0). For the isolation of the enzyme, the cells were centrifuged off at 4000 rpm for 10 min and resuspended in 0.1 M K_2PO_4 buffer, pH 7.4. The ice-cooled cells were carefully disrupted with the aid 30 of a Branson sonifer W25 (Dietzenbach, Germany) at an energy output of 80 W by 2 min sonification three times. The suspensions were centrifuged at 32570 $\times g$ for 20 min. The crude extract was employed for the activity determination or for the enzyme purification. The enzyme purification was carried out as already 35 described in (21), to which reference is expressly made hereby. The concentration of purified enzyme was determined by means of the extinction difference at 450 and 490 nm, as already described in (11), using an extinction coefficient ϵ of 91 $mM^{-1} cm^{-1}$.

40 Example 3:

Isolation of mutants which produce large amounts of blue pigment

100 colonies in each case were isolated from the mutants of one 45 of each position, which were produced by randomized mutagenesis of the codon of the corresponding position. These colonies were cultured in culture tubes for the production of blue pigment.

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After washing the cells with water and a number of slow centrifugation steps (500 rpm), the blue pigment was extracted using dimethyl sulfoxide (DMSO). The solubility of the blue pigment was greatest in DMSO. The absorption of the extract was
5 determined at 677 nm. That mutant which produced the largest amount of blue pigment, especially mutants from a specific position, was used for DNA sequencing (ABI DNA sequencing kit; ABI Prism™ 377 DNA sequencer) and moreover as a template for site-specific randomized mutagenesis.

10

Example 4:

Activity test for the indole hydroxylation

- 15 The indole hydroxylation activity was tested in a solution which contained 8 μ l of a 10–500 mM indole solution in DMSO, 850 μ l of tris/HCl buffer (0.1 M, pH 8.2) and 0.6 nmol of P450 BM-3 wild type or mutant in a final volume of 1 ml. The mixture was preincubated for 9 min before the reaction was started by
20 addition of 50 μ l of an aqueous 1 mM solution of NADPH. The reaction was stopped after 20 sec by addition of 60 μ l of 1.2 M KOH. Within 5 to 30 sec (under aerobic conditions), the enzyme products were converted completely into indigo
25 ([$\Delta^{2,2'}$ -biindoline]-3,3'-dione) and indirubin
30 ([$\Delta^{2,3'}$ -biindoline]-2',3-dione). The indigo production was determined by means of its absorption at 670 nm. A calibration curve using pure indigo showed an extinction coefficient of 3.9 mM⁻¹ cm⁻¹ at this wavelength. A linear curve was obtained for indigo production in a reaction time of 40 sec using 0.6 nmol of
30 wild type or P450 BM-3 mutant and 0.05 to 5.0 mM of indole. Indirubin shows a very weak absorption at 670 nm and the amount of indirubin formed was very much smaller than the amount of indigo formed. The formation of indirubin was neglected in the determination of the kinetic parameters. The NADPH consumption
35 was determined at 340 nm and calculated as described (17) using an extinction coefficient of 6.2 mM⁻¹ cm⁻¹.

Example 5:

40 Purification of indigo and indirubin

- After washing the cells with water and repeated centrifugation at 500 g, the blue pellet formed was extracted using tetrahydrofuran (THF). The extract was evaporated almost to dryness and the red
45 pigment was extracted a number of times with 50 ml of absolute ethanol. The residual blue solid was dissolved in THF and analyzed by thin-layer chromatography (TLC). The ethanol solution

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was evaporated and purified by silica gel chromatography (TLC 60, Merck, Darmstadt, Germany; 2 cm x 30 cm) before it was washed with THF and petroleum ether in a ratio of 1:2. The red solution obtained was evaporated and its purity was determined by TLC. The absorption spectra of the blue and of the red pigment were determined in a range from 400 to 800 nm with the aid of an Ultraspac 3000 spectrophotometer (Pharmacia, Uppsala, Sweden). The blue and the red color were moreover analyzed by mass spectrometry and ¹H-NMR spectroscopy.

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Experimental results

1. Increasing the productivity for blue pigment by P450 BM-3 mutagenesis

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Native P450 BM-3 does not have the ability to produce the blue indigo-containing pigment, or the precursor substances 2- or 3-hydroxyindole. In order to be able to prepare a sufficient amount of blue pigment, P450 BM-3 was subjected to evolution in a controlled manner. All mutants which produced the blue pigment were sequenced. It was found that at least one of the following three positions were mutated: Phe87, Leu188 and Ala74. It was therefore assumed that these three positions play a crucial role for the activity of P450 BM-3 in the production of blue pigment. From the structure of the heme domain of cytochrome P450 BM-3, complexed with palmitoleic acid, it is seen that Phe87 prevents the substrate from coming closer to the heme group (14). The mutant Phe87Val shows a high regio- and stereoselectivity in the epoxidation of (14S, 15R)-arachidonic acid (13) and the mutant Phe87Ala shifts the hydroxylation position of ω -1, ω -2 and ω -3 to ω (22). The position 87 was therefore selected as first for the site-specific randomized mutagenesis with the aid of PCR. In tube cultures, 7 colonies were obtained which produced a small amount of blue pigment after induction. The colony which produced the largest amount of the blue pigment was selected for the DNA sequencing. The sequence data showed substitution of Phe87 by Val. The mutant Phe87Val was then used as a template for the second round of site-specific randomized mutagenesis on position Leu188. The structure of the heme domain, complexed with palmitoleic acid, shows that the repositioning of the F and G helices brings the residue Leu188 into direct contact with the substrate (14). This position can therefore play an important role in substrate binding or orientation. After the second screening passage, 31 colonies were observed which produced the blue pigment. The mutant which produced the largest amount of pigment contained the substitutions Phe87Val and Leu188Gln. This mutant was then mutated in position Ala74 in the third passage of

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site-specific randomized mutagenesis. In this case the triple mutant F87L188A74 (Phe87Val, Leu188Gln and Ala74Gly) was obtained, which produced several mg of blue pigment in a 2-liter flask, containing 300 ml of TB medium. This amount was sufficient for the isolation and characterization of the blue pigment.

2. Isolation and identification of the blue pigment

After washing the cells, the residual blue pellet was extracted with THF and analyzed by TLC. The blue pigment was separated into a rapidly migrating blue component and into a more slowly migrating red component. Both components showed exactly the same mobility parameters as the components of a commercial indigo sample.

15

After the purification, the absorption spectra of both components were determined in DMSO. The blue component showed the same spectrum as a commercial indigo sample. The purified blue and red components were each analyzed by mass spectrometry. The mass spectra of both pigments showed a strong molecular ion peak at $m/e = 262$ and two fragment peaks at $m/e = 234$ and 205 (relative intensity in each case 10%). This pattern is typical of indigoid compounds. The elementary composition of these ions was determined by high-resolution mass spectrometry as $C_{16}H_{10}N_2O_2$, $C_{15}H_{10}N_2O$ and $C_{14}H_9N_2$. This is also characteristic of structures of the indigo type. The blue pigment was thus identified as indigo and the red pigment as indirubin. For the confirmation of the structure, 500 MHz 1H -NMR spectra of both pigments were carried out in DMSO- D_6 solution. The results agreed with the literature data (23).

30

3. Production of indigo using isolated enzymes

It is known that indigo is accessible from indole by microbial transformation (24-26). None of these microbial systems, however, contained a P450 monooxygenase. According to the invention, the catalytic activity of the pure enzyme for indole was first determined. The mutant F87L188A74 was mixed with indole. No color reaction could be observed. Only after addition of NADPH to the reaction mixture was the blue pigment formed after approximately 20 min. By adjustment of the pH of the reaction mixture to a value of approximately 11, 30 sec after addition of NADPH, the blue coloration was visible within a few seconds. Control experiments using native P450 BM-3 were always negative, even using increased concentrations of enzyme, indole and NADPH. The blue pigment was extracted using ethyl acetate and analyzed by TLC. The blue pigment again separated into a more rapidly running

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blue component and into a slower running red component. The R_f values and the absorption spectra were identical to those values of the extracts from the fermentation broth. The F87L188A74 mutant of P450 BM-3 is thus an indole hydroxylase.

5

Two routes have previously been described for the enzymatic transformation of indole to indigo. One route is catalyzed by a dioxygenase, the other by a styrene monooxygenase (24, 25). The NADPH stoichiometry is in both cases 2. It was therefore assumed that in contrast to the dioxygenases the mutant F87L188A74 according to the invention hydroxylates indole in only one position to form oxindole (2-hydroxyindole) or indoxyl (3-hydroxyindole).

15 4. Kinetic parameters of indole hydroxylation

Pure samples of the wild-type enzyme P450 BM-3 and of the mutants Leu188Gln, Phe87Val, F87L188 and F87L188A74 were used for the determination of the kinetic parameters of indole hydroxylation. The results are summarized in Table 1 below.

Table 1: Kinetic parameters of the P450 BM-3 mutants for indole hydroxylation

25	Mutants	K_{cat} (S^{-1})	K_m (mM)	K_{cat}/K_m ($M^{-1}s^{-1}$)
	WT	-a)	-	-
	Leu188Gln	n.d.b)	n.d.	n.d.
	Phe87Val	2.03 (0.14)	17.0 (1.0)	119
	F87L188	2.28 (0.16)	4.2 (0.4)	543
30	F87L188A74	2.73 (0.16)	2.0 (0.2)	1365

a) no activity was observed;

b) not determined (activity was too low to be measured)

Even with an excess of purified enzyme and high indole concentration, the wild-type enzyme is not able to oxidize indole. The mutant Leu188Gln shows a low activity. The mutant Phe87Val shows a catalytic activity of $119 M^{-1}s^{-1}$ for indole hydroxylation. The catalytic efficiency of the double mutant F87L188 (Phe87Val, Leu188Gln) increased to $543 M^{-1}s^{-1}$ and was increased to $1365 M^{-1}s^{-1}$ by introduction of the further substitution Ala74Gly. The K_{cat} values increased from Phe87Val to the triple mutant by a total of 35%, while the K_m values decreased approximately by seven-fold. This indicates that Ala74Gly and Leu188Gln are mainly involved in substrate binding.

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For the triple mutant F87L188A74, the indole turnover rate ($K_{cat}=2.73 \text{ s}^{-1}$) was more than ten times higher than for most P450 enzymes (18).

5 Example 6

Hydroxylation of n-octane using modified cytochrome P450 monooxygenase

10 The reactions were carried out using a P450 BM-3 monooxygenase mutant comprising the following mutations: Phe87Val Leu188Gln Ala74Gly

The chosen substrate was n-octane. For the hydroxylation of
15 n-octane, the following aerobic reaction mixture was used:

P450 BM-3 mutant:	17.5 mg (lyophilisate)
Reaction buffer:	9.1 ml (potassium phosphate buffer 50 mM, pH 7.5)
20 Substrate:	50 μ l of a 60 mM solution (in acetone)
Temperature:	25°C

The enzyme lyophilisate was dissolved in 500 μ l of reaction buffer and initially incubated at room temperature with substrate and
25 reaction buffer for 5 minutes. 300 μ l NADPH solution (5 mg/ml) were then added. Addition of NADPH was repeated two more times. The progress of the reaction was monitored by measuring the absorption at 340 nm, which allows the NADPH decrease to be observed. NADPH is added in aliquots of 300 μ l, since too high a
30 concentration of NADPH in the reaction solution would result in inactivation of the enzyme. To isolate the products, the reaction solution was then extracted three times with 5 ml of diethyl ether. The combined organic phases were dried over MgSO_4 and concentrated. The products were then characterized by TLC, GC/MS
35 and NMR.

The GC/MS analysis of the reaction mixture gave the following result:

40 Compound	Rt[min] ¹⁾	Conversion [%]
4-octanol	13.51	37
3-octanol	14.08	47
2-octanol	14.26	16

45 1) Temperature program: 40°C 1 min isothermic / 3°C/min 95°C /10°C/min 275°C; apparatus: Finnigan MAT 95; GC: HP 5890 Series II

22

Split Injector; Column: HP-5MS (methylsiloxane) 30m x 0.25mm;
Carrier gas: 0.065 ml of He/min

No starting material was found.

5

Example 7:

Hydroxylation of aromatics, heteroaromatics and trimethylcyclohexenyl compounds

10

- a) Example 6 was repeated, but using, instead of n-octane, the substrate naphthalene. The products that were identified were 1-naphthol and cis-1,2-dihydroxy-1,2-dihydronaphthalene. 88% of the naphthalene starting material had been converted.

15

Analytic methods for reactions with naphthalene

GC:

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Apparatus: Carlo Erba Strumentazione Typ HRGC 4160 on Column
Injector; Column: DB5 30m x 0.2 mm; Material: 5% diphenyl-
95% dimethylpolysiloxane; Carrier gas: 0.5 bar H₂;
Temperature program: 40°C 1 min isothermic / 10°C/min to 300°C
Rt(1-naphthol) = 16.68

25

NMR:

1-Naphthol and cis-1,2-dihydroxy-1,2-dihydro-naphthalene were identified in the ¹H NMR.

30

- b) Example 6 was repeated but using, instead of n-octane, the substrate 8-methylquinoline. 5-Hydroxy-8-methylquinoline was identified as main product, in addition to other derivatives (product ratio 5:1). 35% of the starting material used had been converted.

35

- c) Example 6 was repeated but using, instead of n-octane, the substrate α-ionone. 3-Hydroxy-α-ionone was identified as main product, in addition to other derivatives (product ratio: 76:24). 60% of the starting material used had been converted.

40

- d) Example 6 was repeated, but using, instead of n-octane, the substrate cumene (isopropylbenzene). Five monohydroxy products and one dihydroxy product were identified. 70% of the starting material used had been converted.

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REFERENCES

1. Yano, T., Oue, S., and Kagamiyama, H. (1998) Proc. Natl. Acad Sci. USA 95, 5511-5515.
5
 2. Zhang, J.-H., Dawes, G., and Stemmer, W. P. C. (1997) Proc. Natl. Acad Sci. USA 94, 4504-4509.
 3. Wan, L., Twitchett, M. B., Eltis, L. D., Mauk, A. G., and
10 Smith, M. (1998) Proc. Natl. Acad Sci USA 95, 12825-12831.
 4. Cronin, C. N. (1998) J. Biol. Chem 273, 24465-24469.
 5. Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead,
15 H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R.,
Holbrook, I J. (1988) Science 242, 1541-1544.
 6. Hedstrom, L., Szilagyi, L., Rutter, W. J. (1992) Science 255,
1249-1253.
20
 7. Tucker, C. L., Hurley, J. H., Miller, T. R., and Hurley, I B.
(1998) Proc. Natl. Acad Sci. USA 95, 5993-5997.
 8. Quemeneur, E., Moutiez, J.-B. C., and Menez, A. (1998) Nature
25 (London) 391, 301-303.
 9. Marsden, A- F. A., Wilkinson, B., Cortes, J., Dunster, N. J.,
Staunton, I Leadlay, P. F. (1998) Science 279, 199-201.
 - 30 10. Chen, R., Greer, A., and Dean, A. M. (1998) Proc. Natl. Acad
Sci. US4 95, 11666-11670.
 11. Boddupalli, S. S., Estabrook, R. W. and Peterson, J. A.
(1990) J Biol. Chem 265, 4233-4239.
35
 12. Capdevila, J. H., Wie, S., Helvig, C., Falck, J. R.,
Belosludtsev, Y.,Truan, G., Graham-Lorence, S. E., and
Peterson, J. A. (1996) J. Biol. Chem 271, 22663-22671.
 - 40 13. Graham-Lorence, S., Truan, G., Peterson, J. A., Flack, J. R.,
WeL S., Helvig, C., Capdevilla, J. H. (1997) J. Biol. Chem
272, 1127-1135.
 14. Li, H., Poulos, T. L. (1997) Nat. Structural Biol., 4,
45 140-146.
-

24

15. Ravichandran, K G., Sekhar, S., Boddupalli, S., Hasemann, C. A., Peterson, J. A., Deisenhofer, 1 (1993) Science 261, 731-736.
- 5 16. Modi S., Sutcliffe, M. J., Primrose, W. U., Lian, L.- Y., Roberts, G. C. K (1996) Nat. Structural Biol. 3, 414-417.
17. Oliver, C.F., ModL S., Primrose, W.U., Lian, L.Y. and Roberts, G.C.K (1997) Biochem. J. 327, 537-544.
- 10 18. Guengerich, F.G. (1991) J. Biol. Chem 266,10019-10022.
19. Cherry, J. R., Lamsa, M. H., Schneider, P., Vind, J., Svendsen, A-, Jones, A., and Pedersen, A. H. (1999) Nature 15 Biotechnology 17, 379-384.
20. Schwaneberg, U., Schmidt-Dannert, C., Schmitt, J., and Schmid, R. D. (1999) Anal. Biochem. 269, 359-366.
- 20 21. Schwaneberg, U, Sprauer, AL, Schmidt-Dannert, C., and Schmid, R. D. J of Chromatogr. A, in press.
22. Oliver, C.F., Modi, S., Sutcliffe, M.J., Primrose, W.U., Lian, L.Y. and Roberts, G.C.K (1997) Biochemistry 36, 25 1567-1572.
23. Hart, S., Koch, KR., and Woods, D.R. (1992) J Gen. Microbiol. 138, 211-216
- 30 24. Murdock, D., Ensley, B.D., Serdar, C. and Thalen, M. (1993) Bio/Technology 11, 381-385.
25. O'Connor, ICE., Dobson, A-W. and Hartmans, S. (1997) Appl. Environ. Microbiol. 63, 4287-4291.
- 35 26. Eaton, R. W. and Chapman, P. J. (1995) J Bacteriol. 177, 6983-6988.

40

45

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oxidation of organic substrates

<130> M/40241

<140>

<141>

<160> 9

<170> PatentIn Ver. 2.1

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<211> 3150

<212> DNA

<213> Bacillus megaterium

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aat	tta	ccg	tta	tta	aac	aca	gat	aaa	ccg	gtt	caa	gct	ttg	atg	aaa	96
Asn	Leu	Pro	Leu	Leu	Asn	Thr	Asp	Lys	Pro	Val	Gln	Ala	Leu	Met	Lys	
			20					25					30			
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Ile	Ala	Asp	Glu	Leu	Gly	Glu	Ile	Phe	Lys	Phe	Glu	Ala	Pro	Gly	Arg	
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gta	acg	cgc	tac	tta	tca	agt	cag	cgt	cta	att	aaa	gaa	gca	tgc	gat	192
Val	Thr	Arg	Tyr	Leu	Ser	Ser	Gln	Arg	Leu	Ile	Lys	Glu	Ala	Cys	Asp	
		50				55						60				
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Glu	Ser	Arg	Phe	Asp	Lys	Asn	Leu	Ser	Gln	Ala	Leu	Lys	Phe	Val	Arg	
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26

gat ttt gca gga gac ggg tta ttt aca agc tgg acg cat gaa aaa aat	288
Asp Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Lys Asn	
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Trp Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala	
100 105 110	
atg aaa ggc tat cat gcg atg atg gtc gat atc gcc gtg cag ctt gtt	384
Met Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val	
115 120 125	
caa aag tgg gag cgt cta aat gca gat gag cat att gaa gta ccg gaa	432
Gln Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Pro Glu	
130 135 140	
gac atg aca cgt tta acg ctt gat aca att ggt ctt tgc ggc ttt aac	480
Asp Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn	
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Tyr Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Thr	
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Ser Met Val Arg Ala Leu Asp Glu Ala Met Asn Lys Leu Gln Arg Ala	
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Asn Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Phe Gln Glu	
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Asp Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg	
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Lys Ala Ser Gly Glu Gln Ser Asp Asp Leu Leu Thr His Met Leu Asn	
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Gly Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Glu Asn Ile Arg	
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Tyr Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly	
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Leu Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu	
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27

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305 310 315	
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Glu Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala	
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Lys Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp	
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Glu Leu Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp	
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Gly Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser	
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Ala Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala	
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Cys Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly	
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Met Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu	
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Ala Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr	
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Glu Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn	
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28

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Gln Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly	
515 520 525	
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Ala Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn	
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Ala Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val	
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tct act ctt tca ctt caa ttt gtc gac agc gcc gcg gat atg ccg ctt	1968
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Ala Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu	
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690 695 700	

29

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Leu Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu	
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Ala His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln	
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755 760 765	
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Ala Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu	
770 775 780	
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Ser Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser	
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850 855 860	
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Ala Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys	
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Thr Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg	
900 905 910	

CA 02380186 2002-01-23

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915 920 925

gga gaa gca cat tta tac ttc ggc tgc cgt tca cct cat gaa gac tat 2832
Gly Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr
930 935 940

ctg tat caa gaa gag ctt gaa aac gcc caa agc gaa ggc atc att acg 2880
Leu Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr
945 950 955

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cag cac gta atg gaa caa gac ggc aag aaa ttg att gaa ctt ctt gat 2976
Gln His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp
980 985 990

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1010 1015 1020

agt gaa gca gac gct cgc tta tgg ctg cag cag cta gaa gaa aaa ggc 3120
Ser Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly
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cga tac gca aaa gac gtg tgg gct ggg taa 3150
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<213> Bacillus megaterium

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Arg Val
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31

Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Val Arg Asp
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Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Lys Asn Trp
85 90 95

Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
100 105 110

Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
115 120 125

Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Pro Glu Asp
130 135 140

Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145 150 155 160

Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Thr Ser
165 170 175

Met Val Arg Ala Leu Asp Glu Ala Met Asn Lys Leu Gln Arg Ala Asn
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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Phe Gln Glu Asp
195 200 205

Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
210 215 220

Ala Ser Gly Glu Gln Ser Asp Asp Leu Leu Thr His Met Leu Asn Gly
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Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Glu Asn Ile Arg Tyr
245 250 255

Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
260 265 270

Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
275 280 285

Lys Ala Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
290 295 300

Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
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32

Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
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Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
 340 345 350

Leu Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
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Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
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Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
 385 390 395 400

Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
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Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
 420 425 430

Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala
 435 440 445

Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr Glu
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Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr
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Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr
 485 490 495

Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln
 500 505 510

Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala
 515 520 525

Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala
 530 535 540

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Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr
 565 570 575

Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys
 580 585 590

33

Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp
595 600 605

Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val
610 615 620

Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser
625 630 635 640

Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu Ala
645 650 655

Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu
660 665 670

Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu
675 680 685

Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
690 695 700

Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
705 710 715 720

Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu Ala
725 730 735

His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
740 745 750

Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
755 760 765

Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
770 775 780

Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
785 790 795 800

Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
805 810 815

Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
820 825 830

Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
835 840 845

Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
850 855 860

34

Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe
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 Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr
 885 890 895
 Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly
 900 905 910
 Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly
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 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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 Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
 945 950 955 960
 His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
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 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
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 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<212> DNA

<213> Synthetic sequence

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<223> Description of the synthetic sequence: PCR primer

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<210> 4

<211> 30

<212> DNA

<213> Synthetic sequence

35

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<223> Description of the synthetic sequence: PCR primer

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30

<210> 5

<211> 34

<212> DNA

<213> Synthetic sequence

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<223> Description of the synthetic sequence: PCR primer

<400> 5

gaagcaatga acaagnnnca gcgagcaaatt ccag

34

<210> 6

<211> 30

<212> DNA

<213> Synthetic sequence

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<223> Description of the synthetic sequence: PCR primer

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ctggatttgc tcgctgnnnc ttgttcattg

30

<210> 7

<211> 41

<212> DNA

<213> Synthetic sequence

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<223> Description of the synthetic sequence: PCR primer

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<210> 8

<211> 40

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40

36

<210> 9

<211> 1049

<212> PRT

<213> Bacillus megaterium

<400> 9

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 20 25 30

Ile Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Arg
 35 40 45

Val Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp
 50 55 60

Glu Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Val Arg
 65 70 75 80

Asp Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Lys Asn
 85 90 95

Trp Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala
 100 105 110

Met Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val
 115 120 125

Gln Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Pro Glu
 130 135 140

Asp Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn
 145 150 155 160

Tyr Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Thr
 165 170 175

Ser Met Val Arg Ala Leu Asp Glu Ala Met Asn Lys Leu Gln Arg Ala
 180 185 190

Asn Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Phe Gln Glu
 195 200 205

Asp Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg
 210 215 220

Lys Ala Ser Gly Glu Gln Ser Asp Asp Leu Leu Thr His Met Leu Asn
 225 230 235 240

37

Gly Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Glu Asn Ile Arg
245 250 255

Tyr Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly
260 265 270

Leu Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu
275 280 285

Gln Lys Ala Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro
290 295 300

Ser Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn
305 310 315 320

Glu Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala
325 330 335

Lys Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp
340 345 350

Glu Leu Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp
355 360 365

Gly Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser
370 375 380

Ala Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala
385 390 395 400

Cys Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly
405 410 415

Met Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu
420 425 430

Asp Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys
435 440 445

Ala Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr
450 455 460

Glu Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn
465 470 475 480

Thr Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly
485 490 495

Thr Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro
500 505 510

38

Gln	Val	Ala	Thr	Leu	Asp	Ser	His	Ala	Gly	Asn	Leu	Pro	Arg	Glu	Gly	515	520	525
Ala	Val	Leu	Ile	Val	Thr	Ala	Ser	Tyr	Asn	Gly	His	Pro	Pro	Asp	Asn	530	535	540
Ala	Lys	Gln	Phe	Val	Asp	Trp	Leu	Asp	Gln	Ala	Ser	Ala	Asp	Glu	Val	545	550	555 560
Lys	Gly	Val	Arg	Tyr	Ser	Val	Phe	Gly	Cys	Gly	Asp	Lys	Asn	Trp	Ala	565	570	575
Thr	Thr	Tyr	Gln	Lys	Val	Pro	Ala	Phe	Ile	Asp	Glu	Thr	Leu	Ala	Ala	580	585	590
Lys	Gly	Ala	Glu	Asn	Ile	Ala	Asp	Arg	Gly	Glu	Ala	Asp	Ala	Ser	Asp	595	600	605
Asp	Phe	Glu	Gly	Thr	Tyr	Glu	Glu	Trp	Arg	Glu	His	Met	Trp	Ser	Asp	610	615	620
Val	Ala	Ala	Tyr	Phe	Asn	Leu	Asp	Ile	Glu	Asn	Ser	Glu	Asp	Asn	Lys	625	630	635 640
Ser	Thr	Leu	Ser	Leu	Gln	Phe	Val	Asp	Ser	Ala	Ala	Asp	Met	Pro	Leu	645	650	655
Ala	Lys	Met	His	Gly	Ala	Phe	Ser	Thr	Asn	Val	Val	Ala	Ser	Lys	Glu	660	665	670
Leu	Gln	Gln	Pro	Gly	Ser	Ala	Arg	Ser	Thr	Arg	His	Leu	Glu	Ile	Glu	675	680	685
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Pro	Arg	Asn	Tyr	Glu	Gly	Ile	Val	Asn	Arg	Val	Thr	Ala	Arg	Phe	Gly	705	710	715 720
Leu	Asp	Ala	Ser	Gln	Gln	Ile	Arg	Leu	Glu	Ala	Glu	Glu	Glu	Lys	Leu	725	730	735
Ala	His	Leu	Pro	Leu	Ala	Lys	Thr	Val	Ser	Val	Glu	Glu	Leu	Leu	Gln	740	745	750
Tyr	Val	Glu	Leu	Gln	Asp	Pro	Val	Thr	Arg	Thr	Gln	Leu	Arg	Ala	Met	755	760	765
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39

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Glu Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile
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995 1000 1005

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SEQUENZPROTOKOLL

<110> BASF Aktiengesellschaft

<120> Neue Cytochrom P450 Monooxygenasen und deren Verwendung zur Oxidation von organischen Substraten

<130> M/40241

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<170> PatentIn Ver. 2.1

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Asn	Leu	Pro	Leu	Leu	Asn	Thr	Asp	Lys	Pro	Val	Gln	Ala	Leu	Met	Lys	
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Ile	Ala	Asp	Glu	Leu	Gly	Glu	Ile	Phe	Lys	Phe	Glu	Ala	Pro	Gly	Arg	
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Val	Thr	Arg	Tyr	Leu	Ser	Ser	Gln	Arg	Leu	Ile	Lys	Glu	Ala	Cys	Asp	
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Glu	Ser	Arg	Phe	Asp	Lys	Asn	Leu	Ser	Gln	Ala	Leu	Lys	Phe	Val	Arg	
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Gln Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Pro Glu	
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Tyr Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly	
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Lys Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp	
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-4-

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 Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
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Met Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val
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 195 200 205

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 210 215 220

Lys Ala Ser Gly Glu Gln Ser Asp Asp Leu Leu Thr His Met Leu Asn
 225 230 235 240

Gly Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Glu Asn Ile Arg
 245 250 255

Tyr Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly
 260 265 270

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Leu Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu
 275 280 285

Gln Lys Ala Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro
 290 295 300

Ser Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn
 305 310 315 320

Glu Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala
 325 330 335

Lys Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp
 340 345 350

Glu Leu Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp
 355 360 365

Gly Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser
 370 375 380

Ala Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala
 385 390 395 400

Cys Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly
 405 410 415

Met Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu
 420 425 430

Asp Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys
 435 440 445

Ala Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr
 450 455 460

Glu Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn
 465 470 475 480

Thr Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly
 485 490 495

Thr Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro
 500 505 510

Gln Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly
 515 520 525

Ala Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn
 530 535 540

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Ala Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val
 545 550 555 560

Lys Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala
 565 570 575

Thr Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala
 580 585 590

Lys Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp
 595 600 605

Asp Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp
 610 615 620

Val Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys
 625 630 635 640

Ser Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu
 645 650 655

Ala Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu
 660 665 670

Leu Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu
 675 680 685

Leu Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile
 690 695 700

Pro Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly
 705 710 715 720

Leu Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu
 725 730 735

Ala His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln
 740 745 750

Tyr Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met
 755 760 765

Ala Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu
 770 775 780

Leu Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr
 785 790 795 800

Met Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser
 805 810 815

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Glu Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile
 820 825 830

Ser Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser
 835 840 845

Val Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile
 850 855 860

Ala Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys
 865 870 875 880

Phe Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu
 885 890 895

Thr Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg
 900 905 910

Gly Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu
 915 920 925

Gly Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr
 930 935 940

Leu Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr
 945 950 955 960

Leu His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val
 965 970 975

Gln His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp
 980 985 990

Gln Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro
 995 1000 1005

Ala Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val
 1010 1015 1020

Ser Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly
 1025 1030 1035 1040

Arg Tyr Ala Lys Asp Val Trp Ala Gly
 1045

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We claim:

1. A cytochrome P450 monooxygenase which is capable of at least one of the following reactions:
 - a) oxidation of optionally substituted N-, O- or S-heterocyclic mono- or polynuclear aromatic compounds;
 - b) oxidation of optionally substituted mono- or polynuclear aromatics;
 - c) oxidation of straight-chain or branched alkanes and alkenes;
 - d) oxidation of optionally substituted cycloalkanes and cycloalkenes;
- where the monooxygenase is derived from cytochrome P450 monooxygenase BM-3 from *Bacillus megaterium* having an amino acid sequence according to SEQ ID NO:2, which has at least one functional mutation in at least one of the amino acid sequence regions 172-224, 39-43, 48-52, 67-70, 330-335, 352-356, 73-82 and 86-88; except the single mutant Phe87Val.
2. A monooxygenase as claimed in claim 1, which has at least one functional mutation in at least one of the sequence regions 73-82, 86-88 and 172-224.
3. A monooxygenase as claimed in claim 1, which has at least one of the following mono- or polyanino acid substitutions:
 - a) Phe87Val, Leu188Gln; or
 - b) Phe87Val, Leu188Gln, Ala74Gly;and functional equivalents thereof which are capable of at least one of the above oxidation reactions.
4. A nucleic acid sequence coding for a monooxygenase according to one of the preceding claims.
5. An expression construct comprising, under the genetic control of regulatory nucleic acid sequences, a coding sequence which comprises a nucleic acid sequence according to claim 4.
6. A vector comprising at least one expression construct according to claim 5.
7. A recombinant microorganism transformed by at least one vector as claimed in claim 6.

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8. A microorganism as claimed in claim 7, selected from bacteria of the genus *Escherichia*.
9. A process for the microbiological oxidation of an N- or S-heterocyclic mono- or polynuclear aromatic compound, which comprises
- 5 a1) culturing a recombinant microorganism which expresses a cytochrome P450 monooxygenase of bacterial origin in a culture medium, in the presence of an exogenous or
- 10 intermediately formed substrate; or
- a2) incubating a substrate-containing reaction medium with a cytochrome P450 monooxygenase of bacterial origin; and
- b) isolating the oxidation product formed or a secondary product thereof from the medium.
- 15 10. A process as claimed in claim 9, wherein the exogenous or intermediately formed substrate is selected from optionally substituted N- or S-heterocyclic mono- or polynuclear aromatic compounds.
- 20 11. A process as claimed in claim 9 or 10, where the monooxygenase is a mutant as claimed in any of claims 1 to 3, including the mutant Phe87Val.
- 25 12. A process as claimed in claim 11, where the mutant has at least one of the following mono- or polyamino acid substitutions:
- a) Phe87Val;
- b) Phe87Val, Leu188Gln; or
- 30 c) Phe87Val, Leu188Gln, Ala74Gly.
13. A process for microbiological oxidation of a compound as defined in claim 1b), c) or d), which comprises
- 35 a1) culturing a recombinant cytochrome P450-producing microorganism as claimed in claim 7 or 8 in a culture medium, in the presence of an exogenous or intermediately formed substrate; or
- a2) incubating a substrate-containing reaction medium with a
- 40 cytochrome P450 monooxygenase as claimed in any of claims 1 to 3; and
- b) isolating the oxidation product formed or a secondary product thereof from the medium;
- 45 where the monooxygenase mutant Phe87Val is not excluded.
14. A process as claimed in claim 13, wherein the exogenous or intermediately formed substrate is selected from:

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- a) optionally substituted mono- or polynuclear aromatics;
- b) straight-chain or branched alkanes and alkenes;
- c) optionally substituted cycloalkanes and cycloalkenes.

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15. A process as claimed in claim 13 or 14, where the monooxygenase is a mutant as claimed in any of claims 1 to 3, including the mutant Phe87Val.

10 16. A process as claimed in claim 15, where the mutant has at least one of the following mono- or polyamino acid substitutions:

- a) Phe87Val;
- b) Phe87Val, Leu188Gln; or
- 15 c) Phe87Val, Leu188Gln, Ala74Gly.

17. A process as claimed in any of claims 9 to 16, wherein, as exogenous substrate, at least one compound selected from the groups a) to d) of compounds defined above is added to a medium and the oxidation is carried out by enzymatic reaction of the substrate-containing medium in the presence of oxygen at a temperature of approximately 20 to 40°C and a pH of approximately 6 to 9, where the substrate-containing medium additionally contains an approximately 10- to 100-fold molar excess of reduction equivalents based on the substrate.

18. A process as claimed in claim 17, wherein, as exogenous substrate, a compound selected from indole, n-hexane, n-octane, n-decane, n-dodecane, cumene, 1-methylindole, α -, β - or γ -ionone, acridine, naphthalene, 6-methyl- or 8-methylquinoline, quinoline and quinaldine is employed.

19. A process for the microbiological production of indigo and/or indirubin, which comprises

- a1) culturing a recombinant microorganism which produces an indole-oxidizing cytochrome P450 in a culture medium, in the presence of exogenous or intermediately formed indole; or
- a2) incubating an indole-containing reaction medium with an indole-oxidizing cytochrome P450 monooxygenase; and
- b) isolating the oxidation product formed or a secondary product thereof from the medium;

20. A process as claimed in claim 19, wherein the indigo and/or indirubin obtained, which was produced by oxidation of intermediately formed indole, is isolated from the medium.

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21. A process as claimed in claim 20, wherein the indole oxidation is carried out by culturing the microorganisms in the presence of oxygen at a culturing temperature of approximately 20 to 40°C and a pH of approximately 6 to 9.
22. A process as claimed in claim 20 or 21, where the monooxygenase is a mutant as claimed in any of claims 1 to 3 including the mutant Phe87Val.
23. A process as claimed in claim 22, where the mutant has at least one of the following mono- or polyamino acid substitutions:
- a) Phe87Val;
 - b) Phe87Val, Leu188Gln; or
 - c) Phe87Val, Leu188Gln, Ala74Gly.
24. A bioreactor comprising an enzyme as claimed in one of claims 1 to 3 or a recombinant microorganism as claimed in one of claims 7 or 8 in immobilized form.
25. The use of a cytochrome P450 monooxygenase as claimed in one of claims 1 to 3, of a vector as claimed in claim 6, or of a microorganism as claimed in claim 7 or 8 for the microbiological oxidation of
- a) optionally substituted N-, O- or S-heterocyclic mono- or polynuclear aromatic compounds;
 - b) optionally substituted mono- or polynuclear aromatics;
 - c) straight-chain or branched alkanes and alkenes; and/or
 - d) optionally substituted cycloalkanes and cycloalkenes,
- where the monooxygenase mutant Phe87Val is not excluded.
26. The use of a microorganism producing indole-oxidizing cytochrome P450 for the preparation of indigo and/or indirubin.

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